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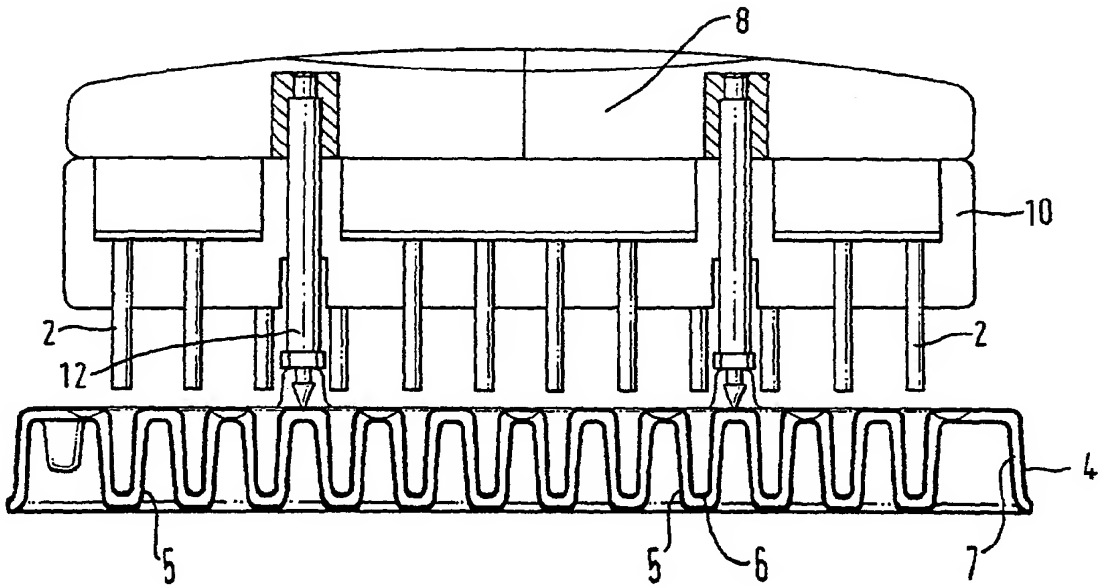
(54) Title: SEPARATION APPARATUS AND METHOD



(57) Abstract: A separation device is disclosed comprising an array of magnetic or magnetisable members (2) and a corresponding array of protective sleeves (6) therefor associated with the magnetic members (2). The magnetic members (2) are removable from the sleeves (6). The sleeves (6) in turn may be removably introduced into the wells (5) of a micro-titre plate (4). Also disclosed is a method of preparing single-stranded DNA (ssDNA) from double-stranded DNA (dsDNA) comprising the steps of immobilising a strand of the dsDNA on a solid magnetic or magnetisable carrier, inserting a magnetic or magnetisable member into a solution containing the DNA to attract said carrier, separating the dsDNA into single strands, and thereafter removing the member from the solution thereby removing ssDNA from the solution.

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Separation Apparatus and Method

This invention relates to an apparatus for separating magnetic elements from non-magnetic elements and methods using such a separation - particularly, although not exclusively for preparing single stranded samples of genetic material.

It is necessary for some methods of analysis of a genetic fragment to separate the two strands of a double-stranded DNA sample. A known method for achieving this comprises amplifying the sample by the Polymerase Chain Reaction (PCR), utilising a biotinylated primer. Paramagnetic beads coated with streptavidin are then added to the sample and allowed to bind to the biotinylated DNA in the presence of a binding buffer. The beads are then separated from the solution by using magnets around the outer walls of the sample container - e.g. the walls of a Micro Titre Plate (MTP) - to hold the beads against the walls of the container whilst a washing buffer is used to flush the liquids away.

The beads are then re-suspended in the washing buffer for the next stage which is the addition of sodium hydroxide which separates the two DNA strands, leaving one still bound to the beads and the other free in the solution. The beads and the bound strands are then held against the walls of the container by the further application of a magnet to the outside of the walls. This allows the sodium hydroxide solution containing the free strands to be removed to a separate container with a pipette, and then to be neutralised with hydrochloric acid. A primer is added and the solution is heated and then cooled, with the result that the primer is annealed to the free strands.

The strands bound to the paramagnetic beads in the

first container are re-suspended in further washing buffer by removing the magnet from the outer wall of the container. A primer is then annealed to these strands as described above.

The above described method can be used successfully but suffers from several drawbacks. Firstly the use of a basic solution to separate the DNA strands necessitates the subsequent addition of an acidic solution in order to neutralise the pH of the sample. However, particularly when the sample is a small volume, this is difficult to achieve accurately and in any event causes contamination of the sample by the resultant salt.

Secondly, the physical removal of the free strands is effected by pipettes. This means that automation is correspondingly complex, particularly where multiple samples are being prepared and the pipettes themselves are a potential source of contamination and must either be replaced or cleaned between uses to avoid contamination of subsequent samples.

Thirdly, at least two washing steps are required which gives a relatively large overall number of steps and also entails the use of relatively large quantities of washing buffer.

Finally the necessity to apply and remove a magnet repeatedly makes the process lengthy.

When viewed from a first aspect, the present invention seeks at least partially to alleviate these drawbacks and provides a method of preparing single-stranded DNA (ssDNA) from double-stranded DNA (dsDNA) comprising the steps of immobilising a strand of the dsDNA on a solid magnetic or magnetisable carrier, inserting a magnetic or magnetisable member into a solution containing the DNA to attract said carrier, separating the dsDNA into single strands, and thereafter removing the member from the solution thereby removing ssDNA from the solution.

Thus in accordance with the invention the segregation of the two separated strands of double-stranded DNA from one another can be effected by removing the magnetic carrier and single-stranded DNA attached thereto from the solution, rather than removing the solution from the carrier, as is the case in the known method. This is advantageous since it allows the easy removal of one of the strands and the easy transport thereof, e.g. into another liquid.

When one strand of the dsDNA is immobilised it may still be attached to the other strand or it may have already been separated therefrom. Thus in some circumstances, where the binding process is specific to one strand, the strand separation may be performed before the immobilisation. Preferably however dsDNA is immobilised on the carrier via one strand thereof, more preferably via one end of one strand thereof (according to techniques well known in the art). Subsequent strand separation thus results in one immobilised strand and one strand free in solution. In either case when the member is removed from the solution, the attracted carrier is also removed having immobilised thereon ssDNA.

The method of the aforementioned aspect of the present invention may be useful in many applications where it is desired to prepare ssDNA. However the method is particularly useful for preparing samples for subsequent analysis using the pyrophosphate-release detection-based sequencing method disclosed in WO 98/13523.

The DNA may be any DNA, e.g. genomic DNA, CDNA or other synthetic DNA molecules, including modified DNA molecules as long as they remain capable of forming DNA.

Methods for immobilisation of DNA are well known in the art. One example of how immobilisation may be conveniently achieved is by binding of biotinylated nucleotides to an avidin/streptavidin-carrying carrier.

Any suitable means can be used for separating the two strands. For example, a base can be used as is used in the known method, whilst retaining at least some of the advantages of the invention set out above. However the method of the invention preferably comprises heating the DNA in order to separate the strands. It will be seen by those skilled in the art that this confers particular benefits because it obviates the disadvantages associated with the use of a base to effect the separation. Thus neutralisation is not necessary with the resultant avoidance of salt contamination.

It will be understood that a method in accordance with the aspect of the invention set out above in its broad terms allows the use of heat denaturing since once denaturing has taken place, physical removal of the ssDNA from the solution can be effected rapidly by withdrawing the magnetic member. This helps to prevent the two strands annealing again on the magnetic carrier. By contrast in the known method where the solution which contains free ssDNA is removed from the container containing the ssDNA attached to the magnetic carrier, it would be necessary to regulate the temperature of the liquid as it was withdrawn in order to prevent annealing. This would be rather difficult to achieve making the use of heat denaturing impractical as an alternative step in the known method.

The method of the invention can of course be carried out singly. However it is preferably carried out in parallel at a plurality of locations. One convenient example of this would be where the dsDNA is held in the wells of a multi-well plate such as an MTP. In order to carry out the method in parallel a plurality of magnetic or magnetisable members is used. Such an apparatus is novel and inventive in its own right and not just in the context of the method described above Therefore when viewed from a second aspect the invention

provides a separation device comprising an array of magnetic or magnetisable members provided on a common support and a corresponding array of protective sleeves therefor associated with the said members.

The present invention also extends to a separation method comprising the use of such an apparatus and to the substances resulting from such a method.

By providing an array of protective sleeves, contact between the magnetic/magnetisable members (referred to collectively hereinafter as "magnetic members", which term is to be understood to include members which are magnetisable but not necessarily magnetic at a given instant) and a substance containing magnetic elements which are to be separated from non-magnetic elements, can be avoided. The sleeves are therefore preferably made from a suitably inert material - e.g. plastics. It is thus possible, in preferred embodiments, to avoid contamination of the magnetic members. The sleeves can be made relatively inexpensively and therefore they may be disposable after use. This reduces the possibility of cross-contamination between consecutive operations of the device.

In a preferred embodiment the magnetic members are separable from the sleeves. This may simply be to allow for their replacement after use as mentioned above. Preferably however the magnetic members are elongate and are received within said sleeves in a longitudinally removable manner. This is particularly useful in the context of permanently magnetic members since it allows the magnetic field at the outer surface of the sleeves (in contact with the working substance in use) to be reduced by withdrawing the magnetic members from the sleeves. When used with the method set out above, such an arrangement allows ssDNA attached to magnetic carriers and thus adhering to the sleeves to be deposited or resuspended by withdrawing the members so

that the carriers are no longer attracted to the sleeves and can thus fall away. This provides a simple mechanism for the selective attraction, manipulation and deposit of e.g. ssDNA bound to magnetic carriers, by moving the members and sleeves as a unit to transport such material and moving the members and sleeves with respect to one another to deposit the material.

A further advantage of the magnetic members and sleeves being mutually removable is that a single array of magnetic members may, if desired, be used with more than one array of sleeves during operation of the device.

The sleeves may be separate from one another, each being coupled individually to the array of magnetic members. Preferably however the sleeves are coupled to one another and the array as a whole is coupled to the array of magnetic members. Most preferably the sleeves are formed integrally with one other e.g. by injection moulding or more preferably, vacuum forming. In accordance with preferred embodiments of the invention the array of sleeves is movably coupled to the array of magnetic members at a plurality of locations so as to give a controlled substantially parallel motion between the two.

The separation device of the present invention finds particular beneficial application in the parallel processing of a plurality of liquid samples contained in an array of wells and thus when viewed from a further aspect the invention provides an apparatus for performing a magnetic separation process on a plurality of samples comprising a separation device having an array of magnetic or magnetisable members provided on a common support and a corresponding array of protective sleeves therefor associated with said members; said apparatus further comprising a plurality of receptacles for containing said samples, the apparatus being arranged such that the members and sleeves may be

removably introduced into said receptacles.

In accordance with this aspect of the invention therefore, magnetic separation within the receptacles can be carried out by inserting therein the magnetic members and sleeves. Magnetic particles from the samples attracted to the sleeves may be removed by removing the sleeves and magnetic members from the receptacles. This allows them to be moved to one or more new receptacles. If, as is preferred, the magnetic members are removable from the sleeves, it can also be arranged that magnetic particles can be selectively attracted to and released from the sleeves.

The apparatus of the invention may be adapted so as to be manipulable by hand. Alternatively or additionally however it may be manipulated automatically by a motor-driven machine or the like.

There may be more or fewer receptacles than magnetic members depending upon the use to which the apparatus is to be put. Preferably at least some of the receptacles are provided integrally on a plate or the like, e.g. a Micro Titre Plate, which enables them to be manipulated easily, the number of receptacles on the plate or the like preferably corresponding to the number of magnetic members.

The apparatus of this aspect of the invention may comprise a single such plate. Preferably however it comprises means to receive a plurality of such plates and means to move the common support for the magnetic members relative to the plates received in said means. This could of course mean that either or both of the common support or the plate receiving means is able to move. In a further preferred embodiment means are provided associated with at least one of the plate receiving locations, for controlling the temperature of the receptacles of a plate received there.

In some preferred embodiments at least one plate receiving location is provided with means to agitate the contents of plates received thereon. This allows the

contents to be mixed for example.

The above features can give rise to an apparatus which is very flexible and which allows an entire series of steps in a separation process to be automated - e.g. with the array of magnetic members being moved successively from one plate to another, the plates being heated, cooled or agitated if necessary.

It may be necessary or desirable in some circumstances to fill the receptacles with such a volume of liquid that they would overflow if the sleeves were to be inserted all the way into them. In order to guard against this, the apparatus is preferably provided with means to limit the extent to which the sleeves can be inserted into the receptacles. This function could for example be provided by a mechanism for driving movement of the sleeves. Additionally or alternatively however a physical stop is provided between the array of sleeves and the receptacles. This gives the function regardless of whether manual or automatic manipulation is used. The stop may be provided adjacent the receptacles - e.g. as a formation on a plate thereof. Preferably however the stop is formed on the array of sleeves, most preferably as an integral protrusion therefrom.

Furthermore the relative dimensions of the sleeves and receptacles are preferably such as to allow for lateral agitation without spillage and correspondingly the apparatus is preferably provided with means to provide lateral agitation - e.g. by vibrating the magnetic members or the receptacles.

The magnetic members in accordance with the invention may be of the temporary type - e.g. a switchable electromagnet whose magnetic field can be varied or switched by controlling the electric current thereto. Preferably however the members comprise a permanent or semi-permanent magnet which enables an apparatus incorporating them to be simple and manufactured relatively inexpensively.

A preferred embodiment of the present invention

will now be described, by way of example only, with reference to the accompanying drawings in which:

Fig. 1 is a cross-section through an array of magnets and sleeves corresponding to an embodiment of the invention;

Fig. 2 is a cross-section similar to Fig. 1, but with the magnetic members withdrawn from the sleeves;

Fig. 3a is a plan view of the sleeve array of Figs. 1a and 1b;

Fig. 3b is a section on line A-A of Fig. 3a;

Fig. 3c is an enlarged portion of part of Fig. 3b;

Fig. 3d is a section on line B-B of Fig. 3a;

Fig. 3e is a section on line C-C of Fig. 3a; and

Fig. 3f is a section on line D-D of Fig. 3a.

Turning to Figs. 1 and 2, there may be seen an array of magnetic members in the form of rods 2, a cover plate 4 comprising an array of sleeves 6, and a mounting block 8. Each of the sleeves 6 is inserted into a corresponding well 5 of a Micro Titre Plate 7. The magnetic rods 2 are mounted to a common supporting member 10 which is itself slidably mounted to the mounting block 8 by means of a plurality of shafts 12 (two of which may be seen in Figs. 1 and 2). The support 10 for the magnetic rods is somewhat flexible as will be explained below. The shafts 12 are mounted at their upper ends in the mounting block 8 and at their lower end they mount the cover plate 4 by means of mounting bosses 14 which are integrally formed in the cover plate. The cover plate 4 is vacuum formed from polystyrene.

In the position shown in Fig. 1a, each of the magnetic rods 2 is received in a corresponding sleeve 6 in the cover plate 4. This means that there will be a significant magnetic field at the outer surfaces of the sleeves which will therefore be felt in the wells 5 of the MTP. If however the common support 10 for the magnetic rods is squeezed along its longitudinal axis, it will arch in the centre and therefore be pushed

upwardly along the shafts 12. When the support 10 is released it will be in the position shown in Fig. 1b, with the rods 2 withdrawn from the sleeves 6, thereby removing the magnetic field from the outer surfaces of the sleeves.

In use the whole assembly depicted is held above a platform which is designed to receive several Micro Titre Plates (MTPs). The assembly may then be moved by hand so that it may be positioned over any of the MTPs.

The cover plate 4 may be seen in greater detail in Figs. 3a to 3f. From these Figures it will be seen that the cover plate has 96 sleeves in a twelve by eight array to match the 96 wells in a standard MTP. However it is not of course necessary that it be used with 96 well MTPs - it could be used on a portion of a larger plate or on a smaller plate, in which latter case the sleeves 6 outwardly of such a plate would not be inserted into wells.

A further feature of interest is seen particularly from Fig. 3f. Between some pairs of sleeves 6 of the cover plate 4 are provided downwardly protruding dimples 16. The function of these is to ensure that the sleeves 6 cannot be inserted so far into the wells of an MTP as to cause the liquid contained in these wells to be pushed out.

An exemplary use of the apparatus described above will now be described. It will be understood however that the use of the apparatus is not limited to this method and it may have many other possible uses.

The method herein set out is for preparing a ssDNA sample for genetic analysis using the method in WO98/13523.

Firstly double-stranded DNA of interest which has been amplified using the Polymerase Chain Reaction wherein the primer is biotinylated is placed inside the wells of an MTP. Paramagnetic beads available under the trade name Dynabeads which are coated with streptavidin are then added to the wells along with a buffered

aqueous solution. Double-stranded DNA in the sample is then allowed to bind to the magnetic beads through the attraction between the biotin and the streptavidin.

A magnetic rod 2 covered by a sleeve 6 is lowered into each well 5 of the MTP 7. The magnetic fields of the rods 2 cause the magnetic beads to be attracted to them and thus cling to the outer surfaces of the sleeves 6. The rods 2 and cover plate 4 are then lifted away from the MTP 7 with the double DNA strands attached to the magnetic beads which still cling to the sleeves 6. The rod array is then translated across to another MTP, the wells of which contain a washing buffer. The rods 2 and sleeves 6 are lowered into this second MTP in order to wash off any liquid contamination thereon. The rods 2 and sleeves 6 are then lifted from the washing buffer and into the wells of a third MTP. This third MTP is disposed on a heating block and its wells contain a buffered aqueous solution and a primer which are heated to approximately 95°C. When the rods 2 are inserted into these wells, the temperature of the solutions therein separates the two DNA strands. This leaves one strand attached to the beads clinging to the sleeves, and the other strand free within the solution. The rods 2 and sleeves 6 are kept in the 95°C solution for between 15 and 60 seconds before being removed again. This gives enough time for the majority of the dsDNA to be separated without allowing an opportunity for the liberated strands to anneal again with strands held to the sleeves 6.

Once the rods 2 and sleeves 6 have been removed from the third MTP, the primer is allowed to anneal with the free strands therein by lowering the temperature. Once the annealing has taken place, the sample is ready to be analysed by the methods set out in WO98/13523.

The removed rods 2 and sleeves 6 are inserted into the wells of a fourth MTP, also containing buffer and a primer and also located on a heating block so as to be heated to approximately 95°C. Once the rods 2 and

sleeves 6 have been inserted into the wells on the fourth plate, the rods 2 are withdrawn from the sleeves 6 by squeezing the support 10 upwardly along the shafts 12, and the sleeves are agitated within the wells by agitating the mounting block 8. The magnetic beads are thus no longer attracted to the sleeves and the agitation causes them to fall off, thereby suspending them in the buffer and primer solution. The solution is allowed to cool so as to allow the DNA strands attached to the beads to anneal with the primer. The samples in this fourth plate are thus also ready for analysis in the same way as those in the third plate.

It will be appreciated by those skilled in the art that modifications to the described embodiment and example may be made without departing from the scope of the invention. For example, the magnetic rod could be an electromagnet rather than a permanent magnet, in which case the relative movement of the cover plate and the rods would not be required since the magnetic field could be switched on or off. Furthermore rather than being manually manipulated, the apparatus could be automated e.g., by providing suitable stepper motors or the like for moving the mounting block and support and an X-Y translation system for moving the assembly laterally.

Claims:

1. A separation device comprising an array of magnetic or magnetisable members provided on a common support and a corresponding array of protective sleeves therefor associated with the said members.

2. A device as claimed in claim 1 wherein said sleeves are made from an inert material.

3. A device as claimed in claim 1 or 2 wherein said sleeves are coupled to one another.

4. A device as claimed in claim 3 wherein the array of sleeves is movably coupled to the array of magnetic members.

5. An apparatus for performing a magnetic separation process on a plurality of samples comprising a separation device having an array of magnetic or magnetisable members provided on a common support and a corresponding array of protective sleeves therefor associated with said members; said apparatus further comprising a plurality of receptacles for containing said samples, the apparatus being arranged such that the members and sleeves may be removably introduced into said receptacles.

6. A device as claimed in any preceding claim wherein said magnetic members are separable from said sleeves.

7. An apparatus as claimed in claim 5 or 6 wherein at least some of said receptacles are provided integrally on a plate or the like.

8. An apparatus as claimed in claim 7 comprising means for receiving a plurality of said plates and means for

moving the common support for the magnetic members relative to plates received within said means.

9. An apparatus as claimed in claim 7 or 8 comprising means for controlling the temperature of said receptacles.

10. An apparatus as claimed in any of claims 7 to 9 comprising means for agitating the contents of said plate.

11. An apparatus as claimed in any of claims 5 to 10 comprising means for limiting the extent to which the sleeves can be inserted into the receptacles.

12. An apparatus as claimed in claim 11 wherein the array of sleeves comprises a stop formed thereon.

13. An apparatus as claimed in any of claims 5 to 12 wherein said sleeves and receptacles are so sized as to allow lateral agitation without spillage from the receptacles when the latter are filled to their maximum predetermined level.

14. A device or apparatus as claimed in any preceding claim wherein said magnetic members comprise a permanent or semi-permanent magnet.

15. A method of separating two substances comprising using a device or apparatus as claimed in any preceding claim.

16. A method of preparing single-stranded DNA (ssDNA) from double-stranded DNA (dsDNA) comprising the steps of immobilising a strand of the dsDNA on a solid magnetic or magnetisable carrier, inserting a magnetic or magnetisable member into a solution containing the DNA

to attract said carrier, separating the dsDNA into single strands, and thereafter removing the member from the solution thereby removing ssDNA from the solution.

17. A method as claimed in claim 16 comprising immobilising dsDNA on the carrier via one strand thereof.

18. A method as claimed in claim 16 or 17 comprising heating the DNA in order to separate the strands.

19. A method of preparing ssDNA from dsDNA comprising carrying out the method as claimed in claim 16, 17 or 18 in parallel at a plurality of locations.

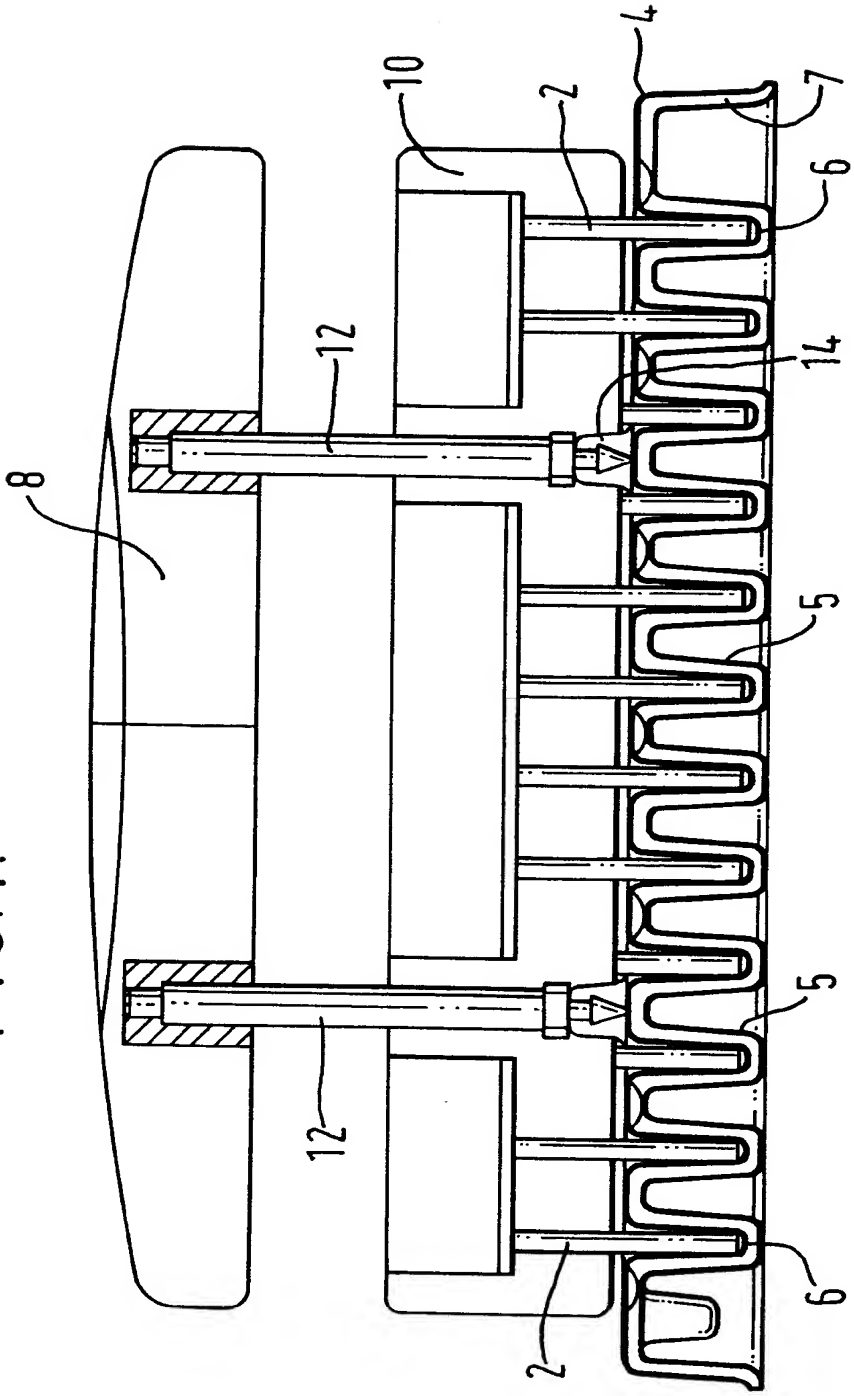

FIG.1.

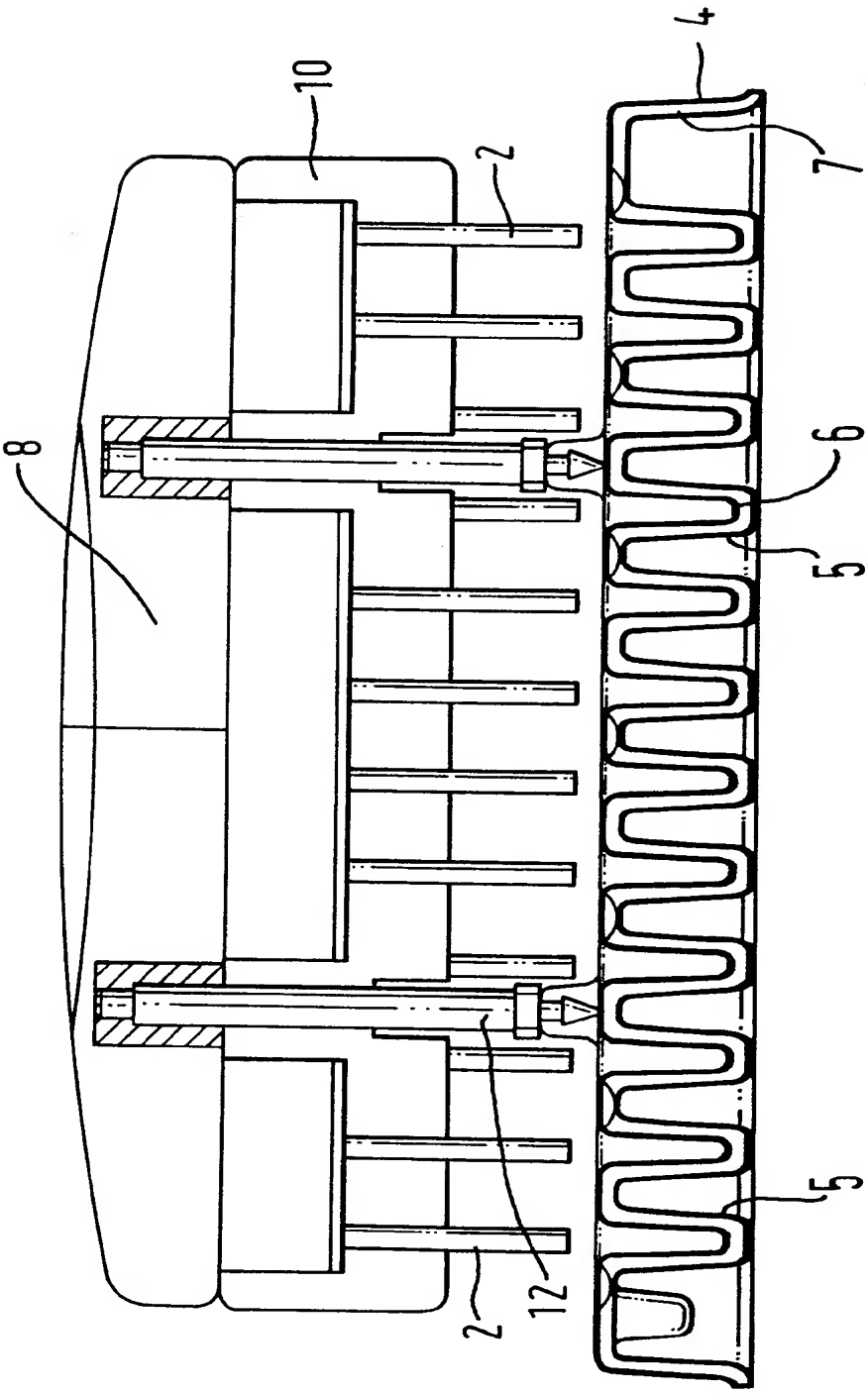

FIG. 2.

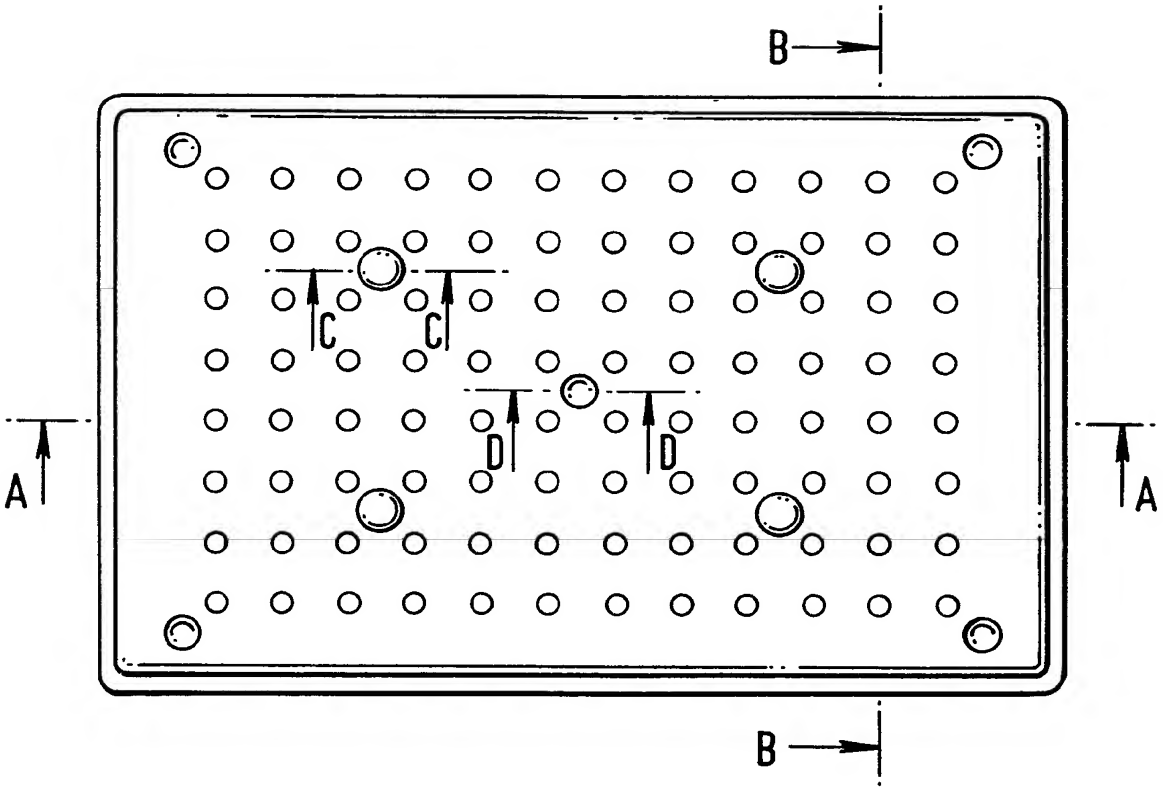

FIG. 3a.

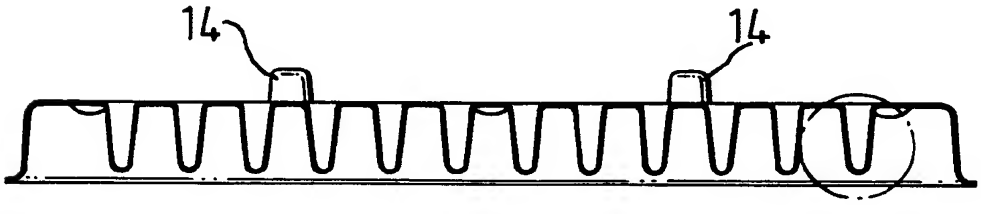

FIG. 3b.

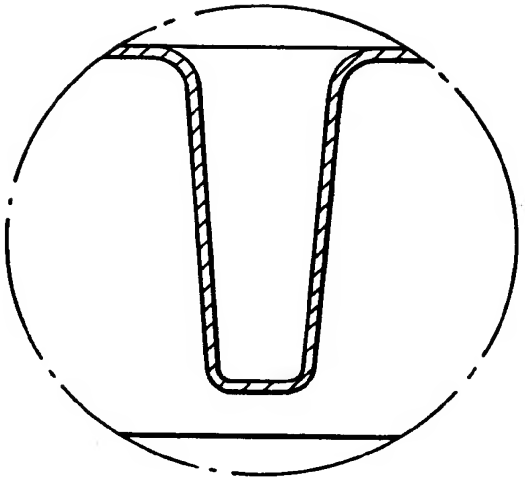

FIG. 3c.

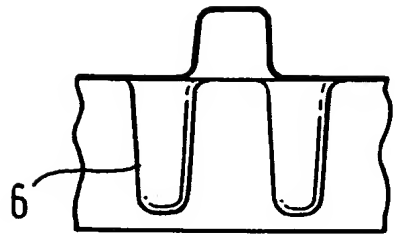

FIG. 3e.

FIG. 3d.

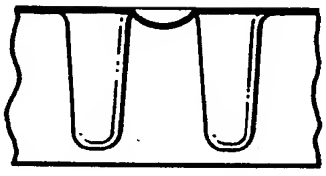

FIG. 3f.